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Ganoderma leucocontextum, a new record from Pakistan

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Abstract—*Ganoderma leucocontextum* is reported from Khyber Pakhtunkhwa province as a new record from Pakistan. This species in the *G. lucidum* complex is characterized by reddish brown to yellowish brown zones on the pileus. The Pakistani specimens are morphologically described and illustrated and their identification supported by a nrITS DNA based phylogeny.

Key words—coniferous, *Ganodermataceae*, polypore, taxonomy, temperate regions

Introduction

Ganoderma P. Karst. (*Ganodermataceae*, *Polyporales*) is a genus of c. 80 accepted species and has a world-wide distribution (Richter & al. 2015). *Ganoderma leucocontextum* is popularly known as a health-oriented herbal mushroom (Li & al. 2015; Wang & al. 2015) and commonly called 'Zanglingzhi' in Tibet Autonomous Region (Wang & al. 2015). The species contains numerous bioactive secondary compounds (Chen & al. 2018) that are pharmacologically valuable in America, Europe, and China due to their therapeutic effects (Xing & al. 2016). However, there has been little scientific research on *G. leucocontextum* due to its rarity (Wang & al. 2015).

Eight *Ganoderma* species have been reported from Pakistan: *G. colossus* (Fr.) C.F. Baker and *G. lucidum* (Curtis) P. Karst. (Ahmad 1956); *G. ahmadii* Steyaert, *G. applanatum* (Pers.) Pat., *G. flexipes* Pat., *G. resinaceum* Boud., and

G. tornatum (Pers.) Bres. (Ahmad 1972); and *G. tsugae* Murrill (Irshad & al. 2012). Here we report on a ninth species for the country, *G. leucocontextum*.

Materials & methods

Collection procedures

Samples were collected during the monsoon season (2018) in Pakistan. Macroscopical features (color, shape, surface texture, length, and width) were noted from fresh basidiomata, which were also photographed in the field. Samples were dried using a dehydrator and stored in sealed polythene bags.

Study sites

The specimens of *Ganoderma leucocontextum* were collected from Ayubia National Park, Khyber Pakhtunkhwa, District Abbottabad, and Naran Valley, District Mansehra, Pakistan. Ayubia National Park is located in the western Himalayas, north of Murree and south of Nathiagali (33°51′55″N 73°08′20″E). This area is covered by temperate broadleaf, mixed, and coniferous forest. The average minimum–maximum temperatures are 3–11 °C and the average annual rainfall is 1244 mm (Afza & al. 2018).

Naran Valley lies in northeastern District Mansehra (34°54.26′N 73°38.90′E). Its climate is dry temperate. Snowfall is heavy in the winter, but summers are cool and dry, and the temperature remains below 10 °C during the whole year (Khan & al. 2011).

Morphological characterization

The size, shape, and color of basidiomata were noted. Color descriptions follow Munsell (1975). Microscopic structures were observed from cross sections of tissues that were soaked in KOH (5% w/v), stained with Congo red (1 %), and viewed under a Meiji Techo MX4300H compound light microscope at 100× magnification. At least 30 measurements were made of all the microscopic features. For basidiospores, 50 measurements were taken and presented as length × width (Nagy & al. 2010).

DNA extraction, sequence alignment and molecular phylogeny

Total genomic DNA was extracted from the material following a modified CTAB procedure (Doyle & Doyle 1987). The nuclear ribosomal ITS region was amplified by using primers ITS1 and ITS2 (White & al. 1990). Reaction mixtures (20 µl) contained 0.5 µl template DNA, 8.5 ml distilled water, 0.5 µl of each primer, and 10 ml PCR mix [DreamTaqGreen PCR Master Mix (2X)]. Amplification conditions were 35 cycles of 95 °C for 30 s, 52 °C for 30 s and 72 °C for 1 min, with a final extension at 72 °C for 10 min. Amplified PCR products were purified and sequenced by Tsingke Co. Ltd.

The consensus sequence was generated from both ITS1 and ITS2 in BioEdit vers. 7.2.5 (Hall 1999) and then homology searches were performed at the NCBI web site using BLASTn. Sequences generated during this study were deposited in GenBank. A dataset of 35 ITS sequences (Table 1) from published literature was downloaded from GenBank. Sequences were aligned and edited using ClustalX 2.1

Table 1. *Ganoderma* and *Amauroderma* sequences used in the phylogenetic analysis.

Species	Origin	Voucher	GenBank No.	Reference
G. lingzhi	Japan	WD-2038	EU021456	Wang & al. 2009
	Japan	WD-565	EU021455	Cao & al. 2012
	China	Wu1006-38	JQ781858	Cao & al. 2012
	China	Cui9164	JQ781859	Cao & al. 2012
G. curtisii	USA	CBS 100131	JQ781848	Cao & al. 2012
	USA	CBS 100132	JQ781849	Cao & al. 2012
G. multipileum	China	Dai 9521	JQ781874	Cao & al. 2012
	Taiwan	BCRC 37043	EU021460	Wang & al. 2009
	India	BCRC 36123	EU021459	Wang & al. 2009
G. tropicum	China	Yuan 3490	JQ781880	Cao & al. 2012
	China	Dai 9724	JQ781879	Cao & al. 2012
	Taiwan	BCRC 37122	EU021457	Wang & al. 2009
G. flexipes	China	Wei 5494	JN383979	Cao & Yuan 2012
	China	Wei 5491	JQ781850	Cao & al. 2012
G. sichuanense	China	Cui 7691	JQ781878	Cao & al. 2012
	China	HMAS 42798	JQ781877	Cao & al. 2012
G. resinaceum	England	HMAS86599	AY884177	Li & al. 2015
	Italy	GrTO96	AM906065	Guglielmo & al. 2008
G. leucocontextum	China	GDGM44304	KJ027608	Li & al. 2015
	China	GDGM44303	KJ027607	Li & al. 2015
	China	GDGM44305	KJ027609	Li & al. 2015
	China	GDGM42932	KF372586	Li & al. 2015
	Pakistan	LAH36345	MK713839	This study
	Pakistan	LAH36346	MN134012	This study
G. valesiacum	Europe	CBS 282.33	Z37056	Moncalvo & al. 1995
G. ahmadii	Pakistan	FWP 14329	Z37047	Moncalvo & al. 1995
G. oregonense	USA	ATCC 46750	Z37061	Moncalvo & al. 1995
	USA	ATCC 46750	Z37061	Li & al. 2013
G. lucidum	Sweden	Dai 2272	JQ781851	Cao & al. 2012
	China	Dai 3937	JQ781853	Cao & al. 2012
	England	HMAS 86597	AY884176	Li & al. 2015
G. tsugae	England	CBS 223.48	Z37054	Moncalvo & al 1995
	England	AFTOL-ID 771	DQ206985	Moncalvo & al. 1995
A. rude	Taiwan	GDGM 25736	KF372587	Li & al. 2015

(Larkin & al. 2007) and BioEdit. These sequences were aligned with MAFFT v. 10 (http://mafft.cbrc.jp/alignment/server/index.html; Katoh & Standley 2013) and manually edited at 593 positions to generate a phylogenetic tree representing 15 taxa, with *Amauroderma rude* (Berk.) Torrend selected as outgroup. MEGA version 10.0 was used to run the maximum likelihood phylogenetic analysis with 1000 bootstrap replicates (Tamura & al. 2011); branches with less than 50% bootstrap support were collapsed.

Taxonomy

Ganoderma leucocontextum T.H. Li, W.Q. Deng, Sheng H. Wu,
Dong M. Wang & H.P. Hu, Mycoscience 56(1): 82 (2015) FIGS 1–2

BASIDIOMATA stipitate, verrucose, zonate, inner zone maroon brown (5YR 4/8), outer zone yellowish brown (7.5YR 6/8), margins white; PILEUS 10–18 × 5–9 cm, 2–3 cm thick, glossy, shiny, smooth, reniform, dimidiate to flabelliform, margins thin, obtuse, white, radially wrinkled, blackish red-brown towards stipe (5YR 3/4), flesh soft with hard crust; STIPE 6–7 × 2–3 cm, cylindrical, slightly wrinkled, glossy, fibrous to woody, chocolate brown (5YR 3/4); PORES 114–153 × 222–255 µm, creamy to white, subcircular; TUBES 5–6 mm long, non-stratified, ochraceous (2.5YR 8/4); CONTEXT 2 cm thick, milky cream (2.5YR 8/4), dry, fibrous, corky.

HYPHAL SYSTEM trimitic, comprising 1) generative hyphae (septate, clamped, colorless, thin-walled), 2) skeletal hyphae (thick-walled, colorless, unbranched or few branches with distal end), and 3) binding hyphae (arboriform, colorless, thick-walled, multi-branched). BASIDIOSPORES 10.4–12.2 × 6.4–10.2 µm, broadly ellipsoid, rough, bitunicate; exosporium hyaline, non-pigmented, thick, brown, episporium verrucose, echinulae dark brown; eusporium, highly thick inter-walled pillars, apically truncate, myxosporium end tapered. CHLAMYDOSPORES 8.2–9.5 × 7.5–8.2 µm, broad, round, rough, pigmented, highly-ornamented. CRUSTOHYMENIDERM CELLS 30–58 × 9–11 µm, amyloid, thick-walled, clavate, originating from skeletal hyphae, brown; below cutis, thick brownish layer composed of hyaline generative and brown skeletal hyphae.

SPECIMENS EXAMINED—**PAKISTAN**, **KHYBER PAKHTUNKHWA**, **Abbottabad District**, Ayubia National Park, 34.0642°N 73.4072°E, elevation 2400 m a.s.l., gregarious on the older roots of *Pinus wallichiana* A.B. Jacks., 12 August 2018, Aisha Umar ANP1 (LAH36345, GenBank MK713839). **Mansehra District**, Naran Valley, 34.9093°N 73.6507°E, elevation 2450 m a.s.l., scattered in organic soil, on the roots of *Pinus wallichiana*, 20 September 2018, Aisha Umar AY2B (LAH36346, GenBank MN134012).

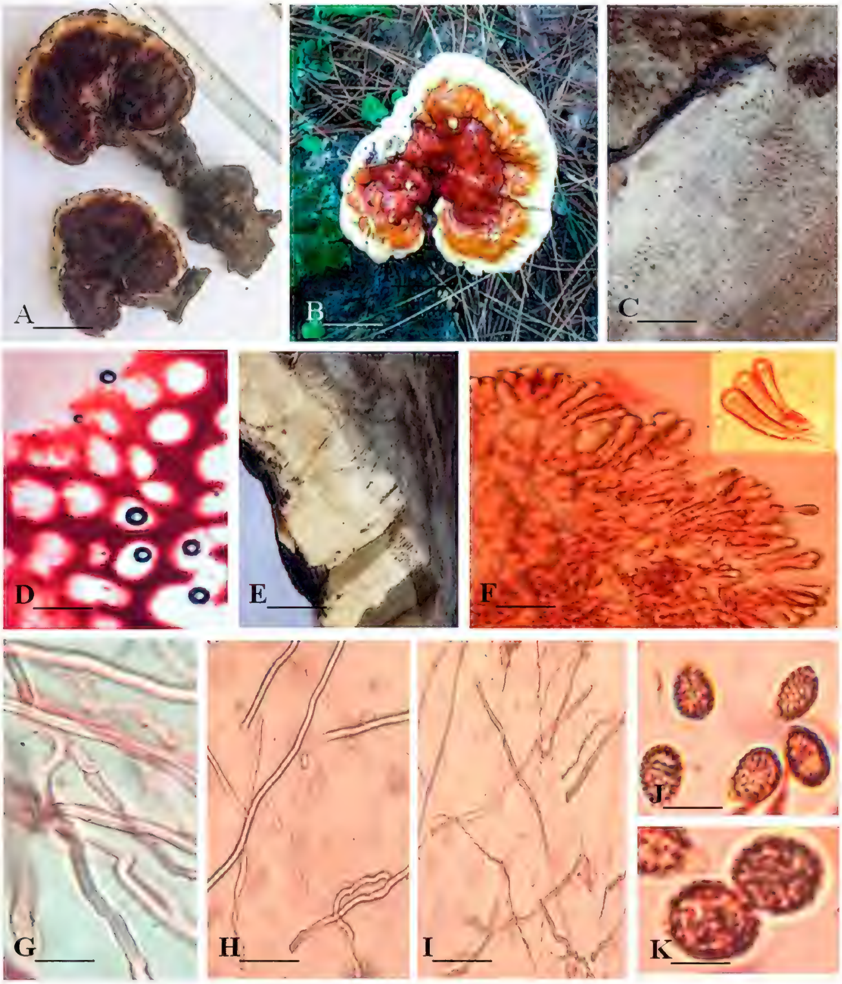

Fig. 1. *Ganoderma leucocontextum* (LAH36345). A, B. Basidiomata; C. Section of context; D. Lower surface pores; E. Section of context; F. Crusto-hymeniderm cells; G. Generative hyphae; H. Skeletal hyphae; I. Binding hyphae J. Basidiospores; K. Chlamydospores. Scale bars: A–C = 2 cm, D = 100 µm, E = 1 cm, G–I = 6 µm; F, J–K = 10 µm.

Molecular phylogeny

The ITS sequence analysis supported our specimen in *G. leucocontextum*. All sequences analyzed clustered in two clades supported by high bootstrap value (Fig. 3). In our tree, *G. leucocontextum* clusters in one clade representing

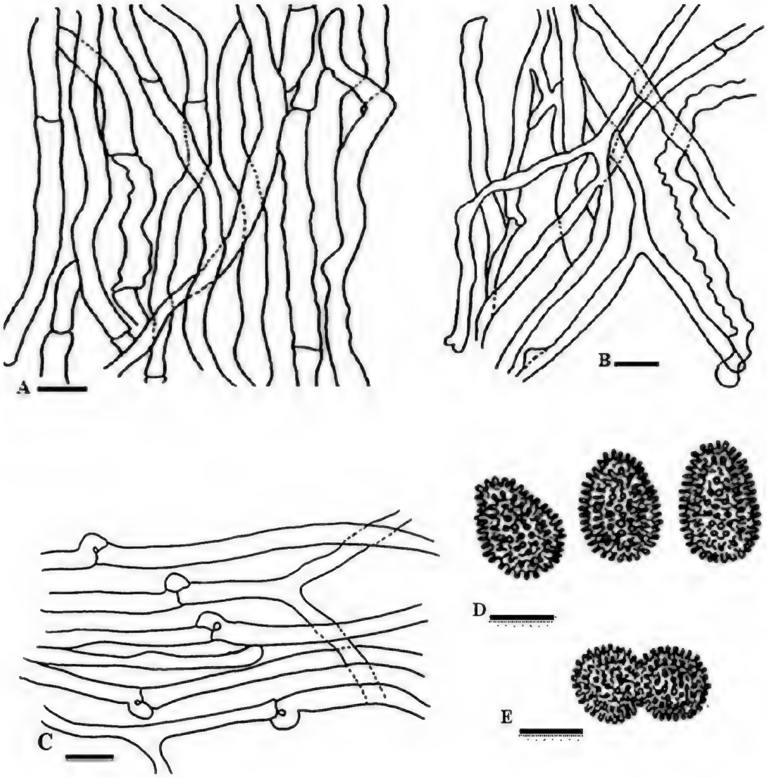

Fig. 2. *Ganoderma leucocontextum* (LAH36345). A, B. Binding and skeletal hyphae; C. Generative hyphae; D. Basidiospores; E. Chlamydospores. Scale bars: A–C = 5 µm; D, E = 10 µm.

G. lucidum, *G. oregonense* Murrill, *G. tsugae*, *G. ahmadii*, and *G. valesiacum* Boud. with 97% bootstrap value. Our specimens (ANP1, AY2B), which nested within the well-supported clade of *G. lucidum* complex, formed a discrete lineage with Chinese *G. leucocontextum* sequences (Li & al. 2015), supported by 96% bootstrap value (Fig. 3).

Discussion

Ganoderma leucocontextum was first described morphologically by Li & al. (2015) based on Chinese material. Our specimens (ANP1, AY2B) and the Chinese specimens both have creamy white context and a long thick

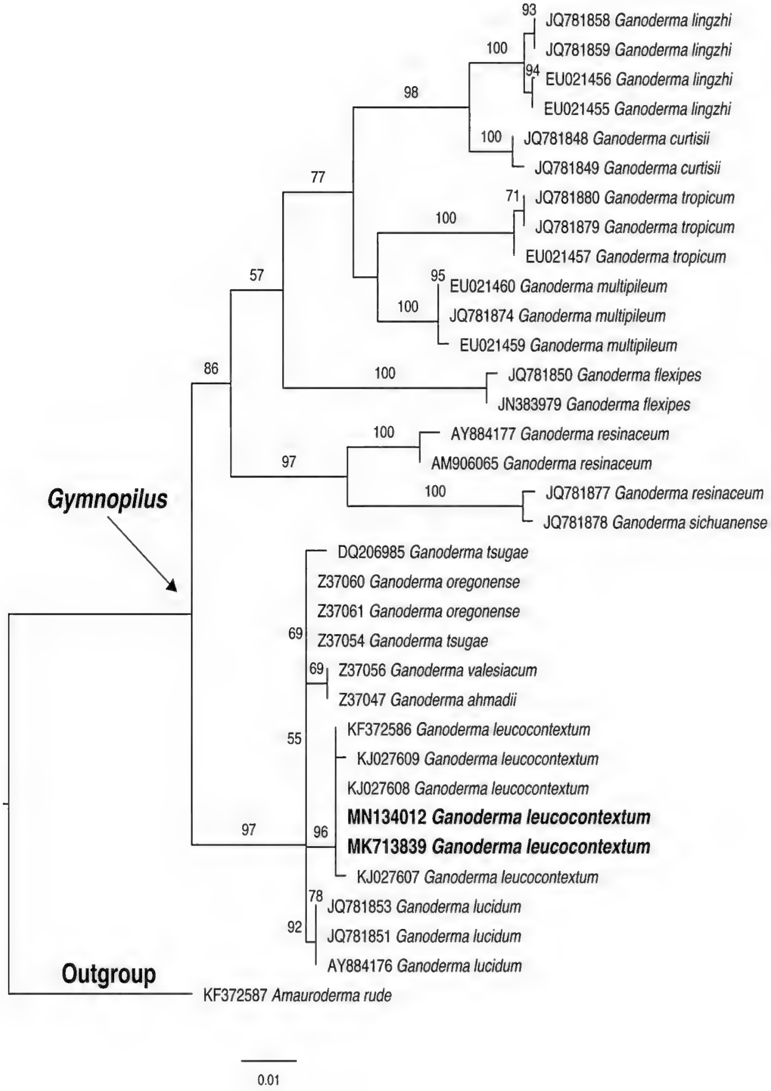
93
JQ781858 *Ganoderma lingzhi*
100
JQ781859 *Ganoderma lingzhi*
94
EU021456 *Ganoderma lingzhi*
98
EU021455 *Ganoderma lingzhi*
100
JQ781848 *Ganoderma curtisii*
JQ781849 *Ganoderma curtisii*
77
71
JQ781880 *Ganoderma tropicum*
100
JQ781879 *Ganoderma tropicum*
EU021457 *Ganoderma tropicum*
57
95
EU021460 *Ganoderma multipileum*
100
JQ781874 *Ganoderma multipileum*
EU021459 *Ganoderma multipileum*
86
100
JQ781850 *Ganoderma flexipes*
JN383979 *Ganoderma flexipes*
100
AY884177 *Ganoderma resinaceum*
97
AM906065 *Ganoderma resinaceum*
Gymnopilus
100
JQ781877 *Ganoderma resinaceum*
JQ781878 *Ganoderma sichuanense*
DQ206985 *Ganoderma tsugae*
Z37060 *Ganoderma oregonense*
Z37061 *Ganoderma oregonense*
69
Z37054 *Ganoderma tsugae*
69
Z37056 *Ganoderma valesiacum*
Z37047 *Ganoderma ahmadii*
55
KF372586 *Ganoderma leucocontextum*
KJ027609 *Ganoderma leucocontextum*
KJ027608 *Ganoderma leucocontextum*
MN134012 *Ganoderma leucocontextum*
97
96
MK713839 *Ganoderma leucocontextum*
KJ027607 *Ganoderma leucocontextum*
78
JQ781853 *Ganoderma lucidum*
92
JQ781851 *Ganoderma lucidum*
AY884176 *Ganoderma lucidum*
Outgroup
KF372587 *Amauroderma rude*
0.01

FIG. 3. Phylogenetic tree of *Ganoderma leucocontextum* and related species based on ITS sequences generated by maximum likelihood method in MEGA 10. *Amauroderma rude* was chosen as outgroup. Bootstrap values >50% are shown at the branches.

cylindrical stipitate fruiting body. The hymenophore pores (4–6 pores per mm) of the Chinese *G. leucocontextum* basidiomata were only slightly smaller and the Chinese crustohymeniderm cells (30–60 × 8–10 µm) were like those in Pakistani specimens. Basidiospore size and ornamentation of Pakistani specimens were also similar to those described for the Chinese *G. leucocontextum* (9.5–12.5 × 7–9 µm; Li & al. 2015).

The Pakistani material also possessed the trimitic hyphal system cited by Li & al. (2015) for *G. leucocontextum*. However, there was a significant difference in host association as the Chinese material was found on the base of dead wood stems of *Fagaceae* [*Cyclobalanopsis glauca* (Thunb.) Oerst.; Li & al. 2015], while our specimens (ANP1, AY2B) were found on the exposed older roots of *Pinaceae* [*Pinus wallichiana*].

The Pakistani *G. leucocontextum* basidiomata (ANP1, AY2B) also resembled *G. lucidum*, *G. oregonense,* and *G. tsugae*, but these species were easily distinguished based on microscopic characters and host habitat. The Chinese *G. leucocontextum* resembled East Asian *G. lucidum* (Li & al. 2015), but East Asian, European (Cao & al. 2012; Wang & al. 2012), and Chinese *G. lucidum* differed from one another based on molecular analysis (Yang & Feng 2013).

Ganoderma leucocontextum, *G. lucidum*, *G. oregonense*, and *G. tsugae* all produce hard basidiomata. Hong & al. (2001) suggested that wrinkles on the pileus surface, smaller basidioma size, and number of pores increase the hardness. *Ganoderma oregonense* basidiomata are reniform, flabellate, pulvinate sessile to laterally substipitate (Gilbertson & Ryvarden 1986), while our specimens possessed a long, thick, cylindrical, stipitate, laccate fruiting body as in *G. lucidum* and *G. tsugae.* The pileal surface color of *G. tsugae* (brown) and *G. lucidum* (reddish-brown) closely matched the Pakistani and Chinese *G. leucocontextum* (Cao & al. 2012) in contrast to the bay blackish brown color typical of *G. oregonense* (Steyaert 1980). Basidioma margins were white in both *G. tsugae* (Steyaert 1980) and *G. leucocontextum* whereas margins were folded (and thus brown) in *G. oregonense.*

The creamy context color in *G. leucocontextum* was close to the creamy to pinkish-buff context of *G. oregonense* (Zhou & al. 2015) and *G. tsugae* (Steyaert 1980). but dissimilar to the deep rusty brown context described for the European *G. lucidum* (Ryvarden & Gilbertson 1993). Pores are subcircular in *G. leucocontextum* and *G. lucidum* (Cao & al. 2012), circular in *G. tsugae,* and circular to irregular in *G. oregonense* (Steyaert 1980).

The 5–6 mm long tubes *G. leucocontextum* are virtually the same length as those cited for *G. lucidum* (5–7 mm). Highly echinulate ellipsoid spores of our specimens were larger than the echinulate ovoid spores of European samples of *G. lucidum* (7–12 × 6–8 µm; Wang & al. 2012) and smaller than the ovoid spores of *G. tsugae* (13–15 × 7.5–8.5 µm; Wasser & al. 2006, Hong & Jung 2004) and ovoid to ellipsoid in *G. oregonense* (11–12.5 × 7–8 µm; Zhou & al. 2015). The spore apex is convex in *G. tsugae*, collapsed in *G. lucidum*, and ellipsoid in *G. leucocontextum* and *G. oregonense*. The basidiospore color is light yellowish brown in both *G. oregonense* and *G. tsugae* and brown in *G. leucocontextum* and *G. lucidum* (Steyaert 1980). Trimitic hyphal system is present as in *G. lucidum*, *G. oregonense*, *G. tsugae* (El-Fallal & al. 2015) and our specimens of *G. leucocontextum*.

Ganoderma lucidum, *G. oregonense*, and *G. tsugae* associate only with conifers (Fernando 2008), whereas *G. leucocontextum* grows on both *Angiospermae* (China) and *Gymnospermae* (Pakistan).

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